

Morphological and Karyological Characteristics and Phylogenetic Relationship of *Asarum cordifolium* C. E. C. Fisch. (Aristolochiaceae) Occurring in Myanmar

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To make clear the morphological and karyological characteristics and the phylogenetic relationship of the poorly known species '*Asarum cordifolium*' from Myanmar, we undertook morphological and karyological studies and phylogenetic analyses of 18 taxa including 14 accessions of *Asarum* and *Saruma*. Morphologically, *Asarum cordifolium* appeared to be assigned to section *Asarum* in sharing some floral aspects: connate styles, inferior ovary, free sepals beyond the attachment to ovary, and stamens with longer filaments. However, unlike the most species of the section *Asarum*, the species had foliaceous leaves alternate to each other and vertically oriented rhizomes, and its somatic chromosome number was $2n = 24$. Phylogenetic analyses based on ITS regions indicate that *A. cordifolium* is positioned within the section *Asarum* and is closely related to *A. caudigerum* and *A. cardiophyllum*.

Key words: Aristolochiaceae, *Asarum*, *Asarum cordifolium*, chromosome number, ITS, Myanmar, phylogeny

Asarum L. is a diverse genus of perennial herbs occurring in subtropical to temperate forest floor of the northern hemisphere and it is composed of approximately 100 species. In a recent morphological and molecular systematic study by Kelly (1998) the genus was subdivided into two subgenera and four sections. According to this system, the species having a false calyx-tube and connate styles with terminal stigmas were assigned to the subgenus *Asarum* section *Asarum*. The section *Asarum* is widely distributed in Eastern Asia, Europe and North America, and 14 species were recognized within the section in a recent classification system by Kelly (2001): six in North America, seven in Eastern Asia and one in Europe. However, the species occurring in the Southern Himalayans was overlooked in

the recent taxonomic studies (Kelly 1998, 2001).

In the Southern Himalayans, only a species '*Asarum cordifolium* C. E. C. Fisch.' has been known so far. This species was described on the basis of specimens collected from Assam and northern Myanmar by Mr. Kingdon Ward (Fischer 1930), and recorded in a checklist of Myanmar Flora (Kress *et al.* 2003). Although the species appears to be assigned to the section *Asarum*, detailed morphological features of the species are poorly known so far, and no karyological and phylogenetic investigations have been undertaken. A few years ago, we got seeds of the species collected by Mr. Yasuyuki Watanabe at northwestern region of Putao, northern Myanmar, and recently had a chance to examine its floral and vegetative characters. In con-

trast to other species of the section *Asarum*, the species has remarkably larger leaves and its leaves are deeply cordate at base, as being pointed out by Fischer (1930). Furthermore, it was shown from a preliminary investigation that the species is unique in growth forms including rhizome within the section and the other species of the genus. We wonder whether the species is truly assigned to the section *Asarum* or not.

In earlier investigations (Sugawara 1981, 1991, Cheng & Yang 1983, Kelly 2001), morphological and karyological characters have been used for understanding species relationships of *Asarum* and more recently molecular data provided by ITS characters partially resolved the species relationships within the *Asarum* section *Asarum* (Kelly 1998). As mentioned above, however, detailed morphological investigations including growth form and karyotype analysis have not been undertaken for *A. cordifolium* and no information is available to help to resolve its phylogenetic relationship. In the present study therefore we aim to document the morphological and karyological characteristics and to present the phylogenetic relationship of the poorly known species of *A. cordifolium* from the Southern Himalayans within *Asarum*, especially in the section *Asarum* based on molecular phylogeny.

Materials and Methods

Morphological and karyological analysis

Asarum cordifolium germinated from the seeds collected at northwestern region of Putao, Kachin State of Myanmar in May 10, 2000, was used. For studying its floral morphology, flowers were fixed with FAA and then examined in more detail with the aid of a binocular microscope.

Karyological investigations were made from four plants germinated from the seeds in Botanical Garden of Tokyo Metropolitan University. The methods for karyological investigations are as follows. Root tips were pretreated with 0.002M 8-

hydroxyquinoline solution for about 3 hr at room temperature and fixed with 45% acetic acid solution for 20–25 min. After being macerated in a 1:2 mixture of 45% acetic acid and 1N HCL for 30 sec at 60°C, the root tips were stained with 1% acetic orcein solution for overnight. Subsequently they were squashed in the standard way (Sugawara & Ogisu 1986, 1992). Nomenclature for centromeric position follows Levan *et al.* (1964).

DNA extraction, PCR and sequencing

The leaves of samples were dried and preserved in silica gel. After powdering the leaves using the mixer mill (Vibration Mill type MM300, Retsch, Haan, Germany), total genomic DNA was extracted from 0.01 g of the powder using the DNeasy Plant Mini Kit (Qiagen Inc., Hilden, Germany). The internal transcribed spacer (ITS) region of the 18-26S nuclear ribosomal DNA was amplified by polymerase chain reaction (PCR). The primers used for PCR were ITS5 and ITS4 in White *et al.* (1990). The PCR reaction mixtures contained 50–100 ng template DNA, 5 µL of 10X PCR buffer (Takara, Japan), 0.2 mM of each deoxyribonucleotide, 2.0 mM of MgCl₂, 0.4 µM of each of the primer pairs, and 1.0 U of ExTaq DNA polymerase (Takara) in a total volume of 50 µL. The PCR program started with 3 min at 94°C for initial denaturation, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 2 min. The reactions were then extended by 7 min at 72°C. The PCR products for direct sequencing were excised from 1% agarose gels and purified using a GENECLAN III Kit (Qbiogene, Inc., Carlsbad, CA, USA). Sequencing reactions were carried out using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 3.1 (Applied Biosystems, Inc., Foster City, CA, USA). The sequencing reaction products were purified, concentrated by EtOH precipitation, and then applied to an ABI Prism 3100-avant automated DNA sequencer (Applied

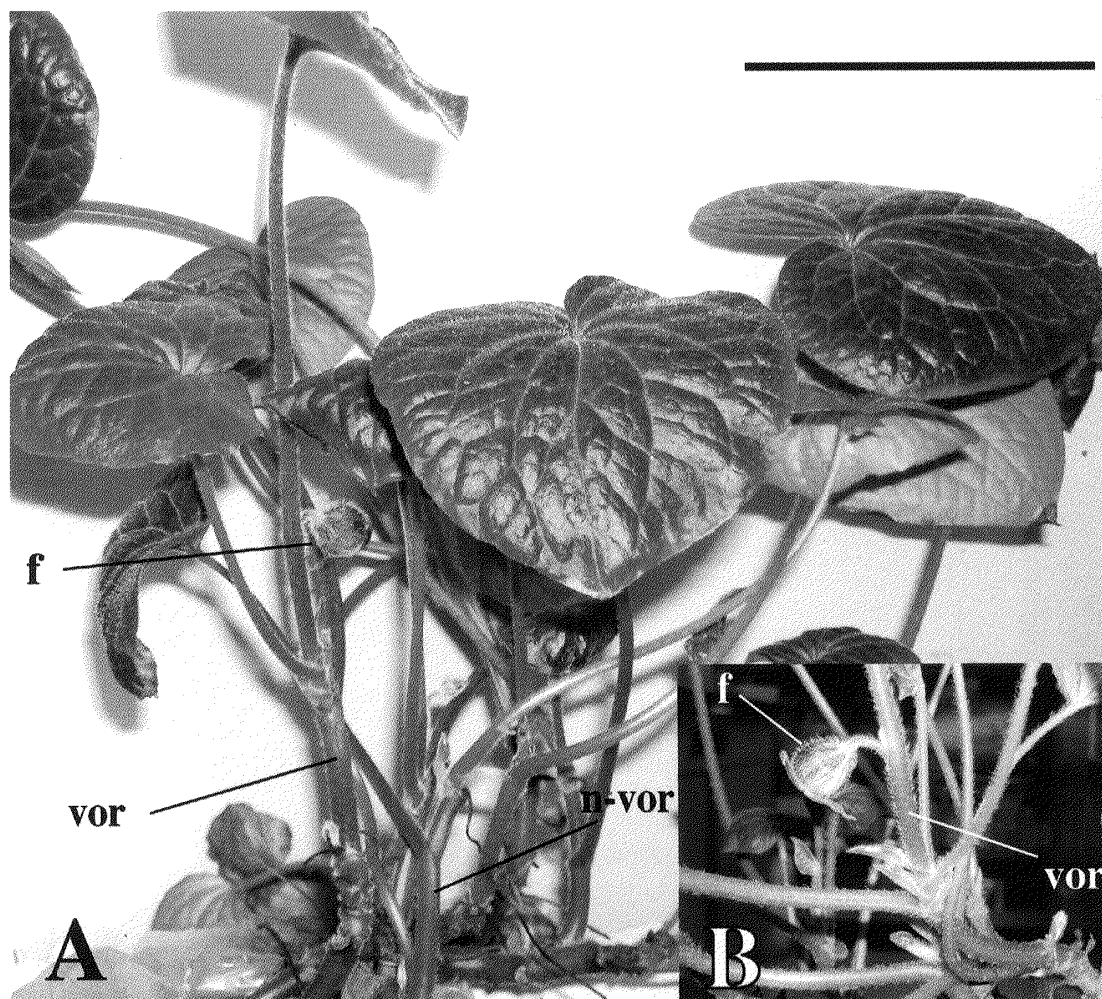


FIG. 1. *Asarum cordifolium*, cultivated in Botanical Garden of Tokyo Metropolitan University. A: Whole plant, showing a seasonal growth along a shoot. B: A flower born at the base of petiole. f, flower. n-vor, vertically oriented rhizome possessing no flower. vor, vertically oriented rhizome possessing a flower. Scale bar = 10 cm.

Biosystems). The obtained sequences have been deposited in the DDBJ, EMBL and GenBank DNA databases (Table 1).

Outgroup selection and phylogenetic analyses

Kelly (1998) performed phylogenetic analysis of *Asarum* based on DNA sequences of nuclear ribosomal internal transcribed spacer (ITS). According to his analysis, section *Asarum*, which appears to include the species concerned here, is sister to section *Geotaenium*. In order to infer the phylogenetic relationships of *A. cordifolium*, thirteen species belonging to the section *Asarum* and two species of the section *Geotaenium* were used (Table 1). We fur-

thermore selected one species of the subgenus *Heterotropa* section *Asiasarum* (*A. sieboldii* Miq.) and one species of *Saruma* (*S. henryi* Oliv.) as outgroups for the phylogenetic analysis (Table 1).

Thirteen nucleotide sequences of ITS region of section *Asarum* previously reported by Kelly (1998) were used. The 22 sequences data including the present new samples (8 accessions) were aligned manually with the DNASIS-Mac program version 3.0 (Hitachi Software Engineering, Tokyo, Japan). Insertions/deletions (indels) were generally placed so as to maximize the number of matching nucleotides in corresponding sequences.

Phylogenetic relationships among the acces-

TABLE 1. *Asarum cordifolium* and other species examined in the present study. Distribution of each species, voucher specimen and accession number are also shown.

Taxon	Distribution, Source (Voucher specimen)	Accession number (ITS1 & ITS2)
<i>Asarum canadense</i> L	North America: Eastern Canada & U.S. A. From Genbank	AF061449 & AF061450
<i>A. cardiophyllum</i> Franch.	Eastern Asia: Southwestern China Mt. Emei, Sichuan, China (MAK362221)	AB219824
<i>A. caudatum</i> Lindl., 1	North America: Western Canada & U. S. A. From Genbank	AF061471 & AF061472
<i>A. caudatum</i> Lindl., 2	North America: Western Canada & U. S. A. Cult. in Tokyo Met. Univ., Japan (MAK197225)	AB219827
<i>A. caudigerum</i> Hance, 1	Eastern Asia: Southwestern China to Taiwan & Japan From Genbank	AF061463 & AF061464
<i>A. caudigerum</i> Hance, 2	Eastern Asia: Southwestern China to Taiwan & Japan Cult. in Tokyo Metropolitan Univ., Japan (MAK362222)	AB219825
<i>A. caudigerellum</i> C.Y. Cheng & C.S. Yang	Eastern Asia: Southwestern China From Genbank	AF061465 & AF061466
<i>A. caulescens</i> Maxim., 1	Eastern Asia: Western China & Japan. From Genbank	AF061459 & AF061460
<i>A. caulescens</i> Maxim., 2	Eastern Asia: Western China & Japan. Mt. Tanzawa, Kanagawa Pref., Japan (MAK219187)	AB219826
<i>A. cordifolium</i> C. E. C. Fisch.	Eastern Asia: Indo. Assam & Myanmar Putao, Kachin State, Myanmar (MAK361296)	AB219828
<i>A. debile</i> Franch	Eastern Asia: China. From Genbank	AF061467 & AF061468
<i>A. epigynum</i> Hayata, 1	Eastern Asia: Southern China & Taiwan. From Genbank	AF061475 & AF061476
<i>A. epigynum</i> Hayata, 2	Eastern Asia: Southern China & Taiwan. Cult. in Tokyo Met. Univ., Japan (MAK362223)	AB219829
<i>A. europaeum</i> L.	Europe: N. France to Caucasus in south, Scandinavia to W. Siberia in north. From Genbank	AF061455 & AF061456
<i>A. hartwegii</i> S. Watson	North America: Western U. S. A. From Genbank	AF061453 & AF061454
<i>A. himalaicum</i> Hook. f. & Thomson	Eastern Asia: Eastern Nepal to southwestern China From Genbank	AF061461 & AF061462
<i>A. lemmonii</i> S. Watson	North America: Western U. S. A. From Genbank	AF061451 & AF061452
<i>A. marmoratum</i> Piper	North America: Western U. S. A. From Genbank	AF061473 & AF061474
<i>A. pulchellum</i> Hemsl.	Eastern Asia: Central to southwestern China. From Genbank	AF061469 & AF061470
<i>A. sieboldii</i> Miquel	Eastern Asia: China & Japan. Narakawa, Nagano Pref., Japan (MAK329505)	AB219830
<i>A. yunnanense</i> T. Sugaw., M. Ogisu & C. Y. Cheng	Eastern Asia: Southwestern China Hekou, Yunnan, China (MAK257702)	AB219831
<i>Saruma henryi</i> Oliver	Eastern Asia: Western to central China From Genbank	AF061552 & AF061553

sions were inferred by the maximum parsimony (MP) method. The most parsimonious trees were obtained by the PAUP* 4.0 program (Swofford 2002) using the branch and bound search option.

The indels characters were ignored for the analyses. The relative levels of support for different clades were estimated using bootstrap analysis (Felsenstein 1985) based on 1000 replicates.

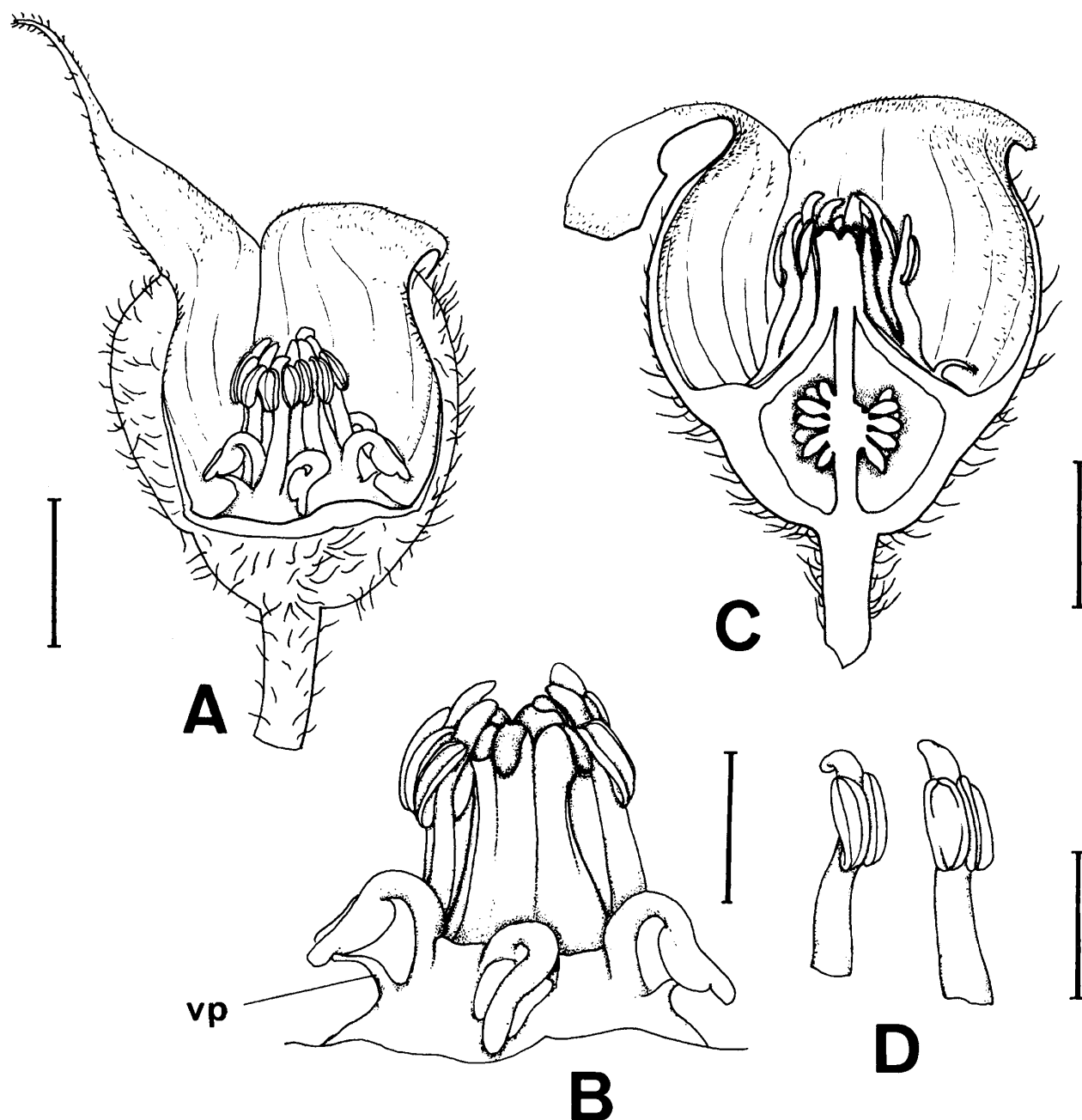


FIG. 2. Illustration of flowers of *Asarum cordifolium*. A: Flower, one sepal removed from the attachment to ovary. B: Stamens and a style column. vp: vestigial petal. C: Longitudinal section of the flower, showing ovary position. D: Stamens with longer filaments. Scale bars = 5 mm (A, C), 2.5 mm (B, D).

Results and Discussion

Morphology and karyomorphology of Asarum cordifolium

Like all species of *Asarum*, *A. cordifolium* has solitary flowers terminating peduncles (Fig. 1). The flowers are actinomorphic and consist of three sepa-

ls, 12 stamens arranged in two whorls of six each and a gynoecium. As in those of the species of the subgenus *Asarum* section *Asarum* (Sugawara 1987, 1991, Kelly 1998), the three sepals of *A. cordifolium* are free but form a spherical calyx-tube beyond the attachment to ovary, and their lobes are spreading horizontally or obliquely and gradually

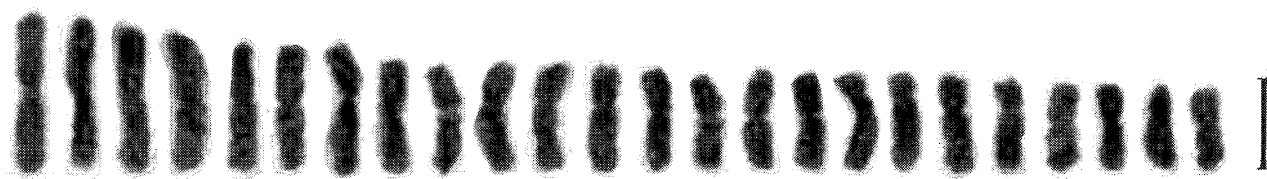


FIG. 3. Serial arrangement of the somatic metaphase chromosomes ($2n = 24$) of *Asarum cordifolium*. Scale bar = 5 μm .

narrowed to form the caudal tips (Fig. 2). Adaxial surface of the calyx-lobes is pubescent and has no sculpture. Filaments of the stamens are obviously longer than the anthers in length. The stamens display a characteristic movement in anthesis; their filaments are recurved and anthers are apart from stigmas before anthesis, but subsequently the filaments gradually erect and locate near the stigmas. The gynoecium consists of six carpels, which are connate to form an inferior-ovary and a stylar-column with six stigmas at the distal tips. Three vestigial petals, which are horn-like in shape, are often found on the inferior ovary (Fig. 2).

In vegetative characters, unlike most of the species of the subgenus *Asarum* section *Aarum*, *A. cordifolium* has vertically oriented rhizomes with relatively longer internodes, and produces two or three cataphylls and a foliage leaf along a shoot for seasonal growth (Fig. 1). Usually species of the section *Aarum* have two foliage leaves on flowering stems, so the leaves appear to be opposite (Cheng & Yang 1983, Sugawara 1987, 1991, Kelly 1998). In contrast, the foliage leaves of *A. cordifolium* are solitary on flowering stem as well as on non-flowering stems so that the leaves are always alternate to each other. Although the leaf of *A. cordifolium* is broadly ovate in shape and cordate at base, as in that of other species of section *Asarum* (Sugawara 1991, Kelly 1998), the leaf blade is more deeply cordate and conspicuously larger, 12–17 cm long \times 10–16 cm wide, and it is densely pubescent on upper surface and sparsely pubescent along veinlets on lower surface.

Somatic chromosome number of *Asarum*

cordifolium was counted as $2n = 24$ for the first time. Within section *Asarum*, the same number has been reported for *A. caudigerum*, *A. cardiophyllum* and *A. pulchellum*, while in the other species another number, $2n = 26$, was observed (Sugawara 1981, 1991, Sugawara & Ogisu 1986, 1992). Of 24 chromosomes, eight have centromeres at submedian region and the others at median region (Fig. 3). No major difference in karyotype was found between *A. cordifolium* and the above three species with the same number $2n = 24$ (see Sugawara & Ogisu 1986, 1992).

Phylogenetic analyses of Asarum cordifolium and other species of Asarum based on ITS sequences

The length of ITS region (ITS1+ITS2) of all accessions varied from 476 to 485 bp in length. The polymorphic characters of the accessions of *Asarum* included 64 nucleotide substitutions and 10 indels. Wagner parsimony analysis of all accessions based on the site changes resulted in 8505 parsimonious trees using the branch and bound search option. The trees required 119 steps; Consistency Index including uninformative characters (CI) = 0.8151, Retention Index (RI) = 0.7843. The strict consensus tree (MP tree) of the 8505 trees is shown in Fig. 4. *Asarum cordifolium* concerned here was nested in a clade of species group of section *Asarum* in the MP tree. This clade was strongly supported as monophyly with 84 % bootstrap probability (BP), and did not include the species of section *Geotaenium* (Fig. 4). The species of *Geotaenium* also formed a monophyletic group (59% BP). Within the large clade of sect *Asarum*, several sub-

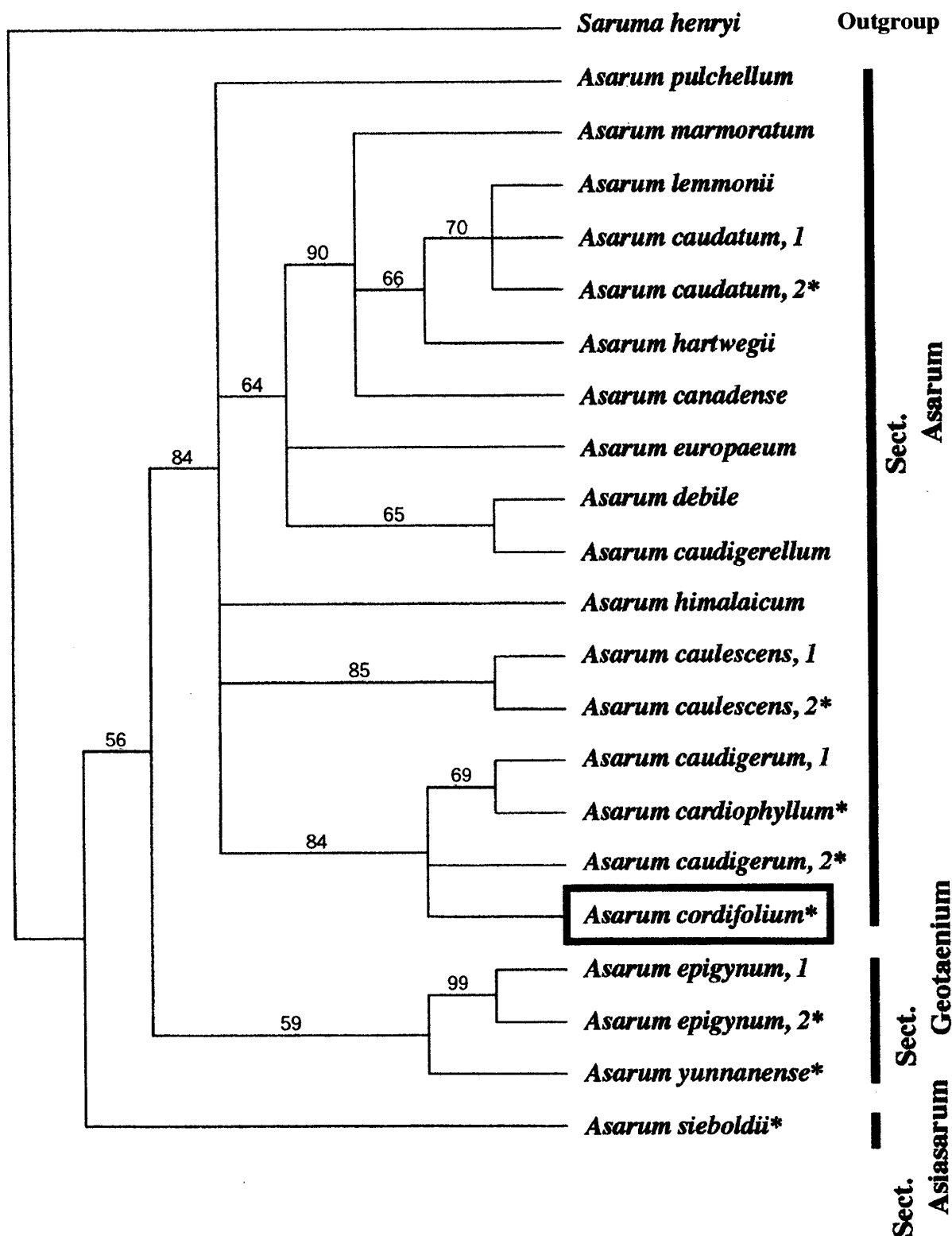


FIG. 4. Strict consensus tree (MP tree) of *Asarum cordifolium* and related taxa, based on ITS regions. Numbers above the branches are the bootstrap values with more than 50% values based on 1,000 replications of the MP analyses. Asterisk (*) following the species name represents the samples, of which DNA sequences of ITS regions are analyzed in the present study.

clades were resolved, but their relationships were not clear. However, it is clear that *A. cordifolium* forms a monophyletic group together with two species of eastern Asia, *A. caudigerum* and *A. cardiophyllum*, with high support value (84% BP).

Morphological comparison and phylogenetic relationships of Asarum cordifolium with other species of Asarum

As just described above, flowers of *Asarum cordifolium* have a false calyx-tube, sepals of which are free from one another beyond the attachment to ovary, and have an inferior ovary and a connate stilar-column with six stigmas at the distal tips. These morphological features suggest that the species belongs to the subgenus *Asarum* section *Asarum* in the classification system by Kelly (1998, 2001). Phylogenetic analysis using the ITS regions indicates that *A. cordifolium* from Myanmar forms a monophyletic group with the species of sect. *Asarum* and thus can be regarded as a member of the section.

Within the section *Asarum*, *A. cordifolium* forms a monophyletic group with *A. caudigerum* distributed widely from southwestern China to Taiwan and Japan and *A. cardiophyllum* in southwestern China, although the detailed phylogenetic relationship among the three species still remains obscure in the present molecular phylogeny. This fact suggests a close relationship of *A. cordifolium* to these two species. Morphologically, *A. cordifolium* is similar to *A. caudigerum* and *A. cardiophyllum* in sharing the calyx-lobes spreading obliquely or horizontally, the apex of which is gradually narrowed to be a slender tip (Kelly, 2001), and in sharing the same chromosome number, $2n = 24$ (Sugawara & Ogisu 1986, 1992). However, the growth form of the former species is clearly different from those of the latter two species. Namely, in *A. cordifolium* the foliage leaves are solitary both on flowering stems and non-flowering ones and thus appear to be alternate, while in the other two species

paired foliage leaves are usually found in all stems and appear to be opposite. Additionally, the leaf size is obviously larger in *A. cordifolium* than in the other two species. The growth form represented by *A. cordifolium* is also found in *A. himalaicum* distributed from eastern Nepal to southwestern China (Kelly, 2001), but the latter species has no vertically oriented rhizomes and it is easily distinguished from *A. cordifolium* by its sepal lobes reflexed and appressed to a false calyx-tube (Kelly 2001) and by its somatic chromosome number, $2n = 26$ (Sugawara 1991). Similarity in the growth form, therefore, may have arisen independently in the two species.

In conclusion, the present study indicated that *Asarum cordifolium* occurring in Myanmar was a member of the subgenus *Asarum* section *Asarum* in the system by Kelly (2001) and, within the section closely related to *A. caudigerum* and *A. cardiophyllum* distributed widely in eastern Asia, but it is distinct from the latter species in some morphological aspects.

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